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APPLICATION NOTE 21764

Accurate and precise quantification of mAb-released *N*-glycans with an amide HILIC column

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Keywords

Inter- and intra-column reproducibility, column lifetime, quantification, released *N*-glycan, Accucore 150 Amide HILIC column, solid core, IgG, Vanquish UHPLC, 2-AB label, APTS label, NIST mAb

Application benefits

- Proven intra- and inter-column reproducibility in retention time, backpressure, and peak shape with overall run-to-run % RSD < 2% and lot-to-lot % RSD < 7%
- Solid core particle results in moderate backpressures with UHPLC efficiencies
- Accurate and precise quantification of human IgG *N*-glycans with absolute average % bias at 3%
- Excellent correlation (R^2 > 0.99 in Deming regression) with a fully validated manufacturing quantification method

Goal

To demonstrate the robustness, reproducibility, accuracy, and precision of quantification of IgG *N*-glycans using the Thermo Scientific™ Vanquish™ Horizon UHPLC integrated biocompatible system and Thermo Scientific™ Accucore™ 150 Amide HILIC column.

Introduction

More than two-thirds of recombinant biopharmaceutical products on the market are glycoproteins, and every stage of their manufacturing is carefully monitored and tested to ensure consistency in quality, safety, and

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effectiveness.[1] Glycosylation is one of the key critical quality attributes (CQAs) of monoclonal antibody (mAb)-based biotherapeutics. Glycosylation changes can impact a biological drug's safety, efficacy, clearance, and immunogenicity, making it necessary to accurately detect changes. Robust, information-rich, and reproducible methods for glycan analysis must be included in regulatory filings for glycoprotein-based biotherapeutics to ensure accuracy and consistency.[2] Current glycan analysis methods involve sample preparation, followed by high-performance capillary electrophoresis (HPCE) or high-performance liquid chromatography (HPLC) separation. Sample preparation takes anywhere from a few hours to multiple days for a 96-well plate. Techniques include protein denaturation, enzymatic deglycosylation, dye labeling, and excess matrix removal. Glycan separation methods typically isolate molecules and their fragments based on different physical properties with HPCE or hydrophilic interaction chromatography (HILIC).

Methods coupling liquid chromatography to fluorescence detection (LC-FLD) have been extensively used for glycan analysis, affording relative quantification of glycans in a sample with good reproducibility.[3] HILIC using amide-based stationary phases is a well-established, robust technique employed by many laboratories to obtain high-resolution separation of *N*-linked glycans released from glycoproteins. Tagging the glycans with a fluorescent label such as 2-AB (2-aminobenzamide), 2-AA (2- anthranilic acid), or APTS (8-aminopyrene-1,3,6-trisulfonic acid trisodium salt) allows the sugars to be detected at femtomole levels. Designed for the separation of hydrophilic biomolecules in HILIC mode, the Thermo Scientific™ Accucore™ 150 Amide HILIC columns are an excellent choice for glycan separations. Based on Core Enhanced Technology™, Accucore columns provide fast, high-resolution biomolecule separations, without the elevated backpressures required by sub-2 µm particles.

Here, the Accucore 150 Amide HILIC column (2.1 × 150 mm) demonstrated great inter- and intra-column reproducibility as well as excellent column lifetime for protein glycan analysis. Furthermore, superb accuracy and precision were obtained for the relative quantification of human IgG *N*-glycans, which is critical for quality control and risk management in biopharmaceutical manufacturing.

Experimental

Consumables and apparatus

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Optima™ acetonitrile (ACN) (A955-4)
- Ammonium formate (99.99% purity) from a reputable supplier

Standards

- 2-AB labeled fetuin *N*-glycan and APT labeled maltotriose were kindly provided by Thermo Fisher Scientific colleagues
- 2-AB labeled human IgG *N*-glycan from a reputable supplier
- APT labeled NIST mAb *N*-glycans were prepared in house with a newly developed *N*-glycan sample preparation kit

Equipment

Vanquish Horizon UHPLC system, including:

- System Base Vanquish Horizon (P/N VH-S01-A)
- Binary Pump H (P/N VH-P10-A)
- Column Compartment H (P/N VH-C10-A)
- Split Sampler HT (P/N VH-A10-A) with 25 µL sample loop
- Fluorescence Detector F (P/N VF-D50-A)

LC conditions

Column	Accucore 150 Amide HILIC 2.1 × 150 mm, 2.6 µm (P/N 16726-152130)
Mobile phase A	ACN 100%
Mobile phase B	Ammonium formate 100 mM, pH 4.4
Flow rate	0.45 mL/min
Column temperature	50 °C
Sample volume	1–10 µL
Mobile phase gradient	Table 1
Fluorescence detector (FLD)	Excitation at 320 nm; Emission at 420 nm for 2-AB labeled glycan Excitation at 455 nm; Emission at 500 nm for APTS labeled glycan

Chromatographic data processing and software: Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.2.5

Table 1. LC gradient

Time (min)	% A	% B
0	70	30
10	68	32
45	40	60
47	40	60
47.5	70	30
50	70	30

Results and discussion

Column lot-to-lot reproducibility test

Column reproducibility is an essential and critical requirement in chromatography. In this study, three random lots of Accucore 150 Amide HILIC columns were evaluated with 2-AB (2-aminobenzamide) labeled fetuin *N*-glycans. Consistent intra- and inter-column performance was achieved (Figure 1A and 1B). Retention time, peak asymmetry, and efficiency were calculated for the 2-AB labeled fetuin glycans A3G3S2 (peak A), A3G3S3 (peak B), and A3G2S4 (peak C) and very compelling results were obtained. As shown in Table 2, the lot-to-lot % RSD for retention time, peak asymmetry, and efficiency are less than 2%, 6.5%, and 3%, respectively. The backpressure was also monitored and was very moderate (around 170 bar, which is 2500 psi). Reproducible pressure traces were observed with column-to-column % RSD < 3% (figures not shown). Overall lot-to-lot reproducibility % RSD < 7%, with protein glycan separations indicating excellent column reproducibility with this HILIC phase.

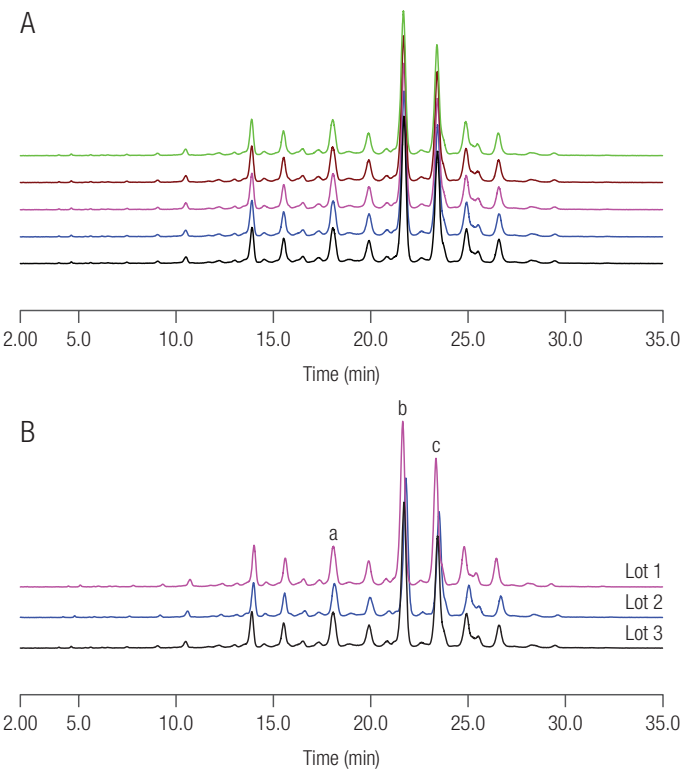


Figure 1. Intra- (A) and Inter- (B) column chromatogram with 2-AB labeled fetuin *N*-glycan

Table 2. Column lot-to-lot reproducibility.

	RT % RSD	Peak Asym. % RSD	Efficiency (EP) % RSD
Peak a: A3G3S2	1.83%	6.39%	3.18%
Peak b: A3G3S3	1.71%	1.09%	2.65%
Peak c: A3G2S4	1.61%	1.64%	1.41%

Column lifetime – robustness test

The column lifetime was evaluated with 500 continuous runs at a 10 µL injection volume with APTS labeled protein glycan/maltotriose across nine days. The APTS labeled samples were aliquoted and stored at -20 °C. Each day of analysis, a fresh aliquot of NIST mAb *N*-glycan sample was removed from the freezer for analysis. Figure 2 shows the NIST mAb *N*-glycan chromatograms from across the 500 injections, demonstrating excellent reproducibility. The retention time of major glycan components in this mAb were calculated and <0.5% RSD was obtained (Table 3). The G1Fa and G1Fb isomer peaks were well resolved; USP resolution was measured from 2.07 to 2.26 with % RSD at 2.76%. The quantification consistency was evaluated with relative area % for these five major components (Table 4). The relative area % for each component (ranging from 2% to 45%) was almost identical from run-to-run with the RSD of all the major peaks at 1% with one low abundant peak at 3%. This durable, dependable and robust performance indicated no trace of column degradation across at least 500 injections of protein glycan separation.

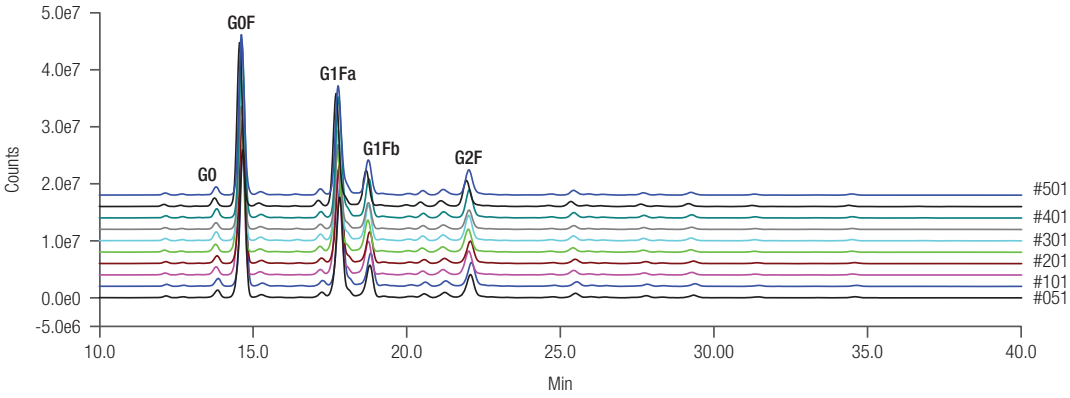


Figure 2. Column lifetime test – chromatogram overlay from injection #1 to #501

Table 3. Column lifetime test – retention time and resolution reproducibility

501 injections	G0	G0F	G1Fa	G1Fb	G2F	Resolution between G1Fa and G1Fb
NIST 1	13.990	14.83	17.990	18.990	22.29	2.07
NIST 51	13.851	14.664	17.810	18.810	22.08	2.15
NIST 101	13.872	14.684	17.851	18.830	22.10	2.14
NIST 151	13.789	14.622	17.768	18.750	22.01	2.15
NIST 201	13.830	14.664	17.809	18.789	22.08	2.14
NIST 251	13.789	14.622	17.747	18.747	21.99	2.22
NIST 301	13.809	14.622	17.768	18.747	22.02	2.23
NIST 351	13.789	14.622	17.768	18.747	22.02	2.25
NIST 401	13.809	14.643	17.789	18.768	22.04	2.26
NIST 451	13.747	14.560	17.705	18.685	21.94	2.24
NIST 501	13.789	14.622	17.768	18.747	22.02	2.20
Average	13.825	14.65	17.798	18.783	22.05	2.18
RSD	**0.48%**	**0.46%**	**0.43%**	**0.43%**	**0.41%**	**2.76**

Table 4. Quantification consistency: major peaks % relative area count of human IgG *N*-glycan

Rel. Area %	G0	G0F	G1Fa	G1Fb	G2F
NIST 1	2.20	45.15	33.45	10.61	8.59
NIST 51	2.20	44.39	33.92	10.63	8.86
NIST 101	2.20	44.28	34.10	10.66	8.76
NIST 151	2.27	44.12	33.91	10.60	8.68
NIST 201	2.27	44.28	34.13	10.60	8.72
NIST 251	2.28	44.12	34.28	10.58	8.74
NIST 301	2.29	43.84	34.47	10.65	8.75
NIST 351	2.29	43.80	34.64	10.66	8.61
NIST 401	2.12	43.75	34.50	10.77	8.86
NIST 451	2.13	43.75	34.63	10.63	8.85
NIST 501	2.13	44.09	34.61	10.36	8.82
Average	2.22	44.14	34.24	10.61	8.75
RSD	**3.0%**	**0.9%**	**1.1%**	**0.9%**	**1.1%**

Quantification of 2-AB labeled human IgG *N*-glycan.
As glycosylated biotherapeutics move through the development pipeline, the glycoprofile and *N*-glycan species present are characterized. As part of this process, the CQAs are monitored closely to ensure production batches remain within defined acceptance criteria, and to identify those parameters that are critical, as part of a quality-by-design (QbD) approach. The *N*-glycan profile is monitored due to the importance of these glycans on the safety and efficacy of the biotherapeutic.

An ideal method for the analysis of *N*-glycans would both identify the isomeric structure and deliver a true picture of the relative, if not absolute, amounts of the various structures in one sample.[4] Correct relative quantitation within a sample requires well-separated peaks, as facilitated by the latest HILIC columns.[5] The Accucore 150 Amide HILIC HPLC column offers excellent separation and quantification of 2-AB labeled human IgG *N*-glycans.

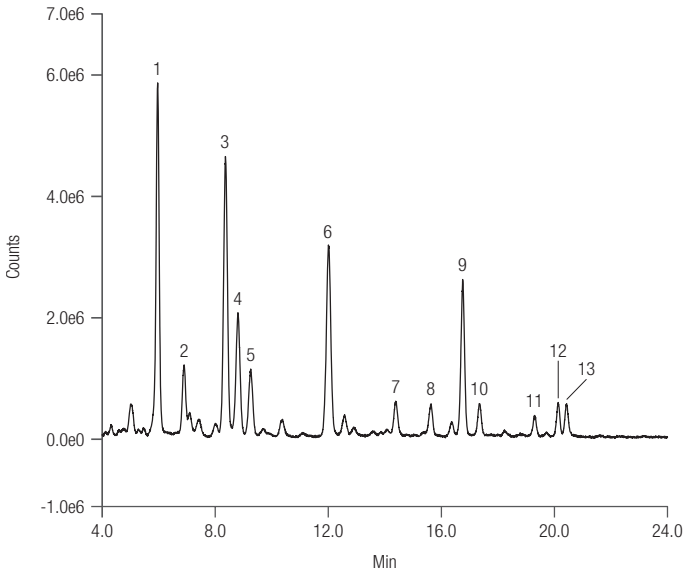


Figure 3. Chromatogram of 2-AB labeled human IgG *N*-glycan by Acuccore 150 amide HILIC column

A representative chromatogram of 10 pmol 2-AB labeled human IgG *N*-glycans (1 µL injection volume) separated on the Acuccore 150 Amide HILIC column and Vanquish Flex UHPLC system can be seen in Figure 3. Thirteen well-resolved major glycans were detected within 22 minutes with moderate backpressure < 200 bar (3000 psi). Peak shapes were excellent for all components, with no significant tailing or asymmetries, and an average peak width at half height (PWHH) under 7 seconds. The % relative peak area of each component glycan was calculated with average % RSDs < 4% (n=5). The quantitative results were compared to the 2-AB labeled human IgG *N*-glycan standard manufacturers' Certificate of Analysis data; details are listed in Table 5.6 With all thirteen tested glycans, the highest variability was F(6)A2BG(4)2 (peak 7) with % bias of + 9%; the lowest was F(6)A2[3]G(4)1 (peak 4) at practically 0% RSD. An absolute average % bias of ± 3% indicates that the test data is highly consistent with manufacturer data. Deming regression (Figure 4) has a slope of 1.017, demonstrating parallelism between the manufacture methods and the Acuccore 150 Amide HILIC-based analysis. A correlation (R^2) of > 0.99 indicates an excellent correlation between the results obtained in the two analyses. Overall, great accuracy and precision were obtained when this Accucore Amide HILIC column is used for the quantification of human IgG *N*-glycans.

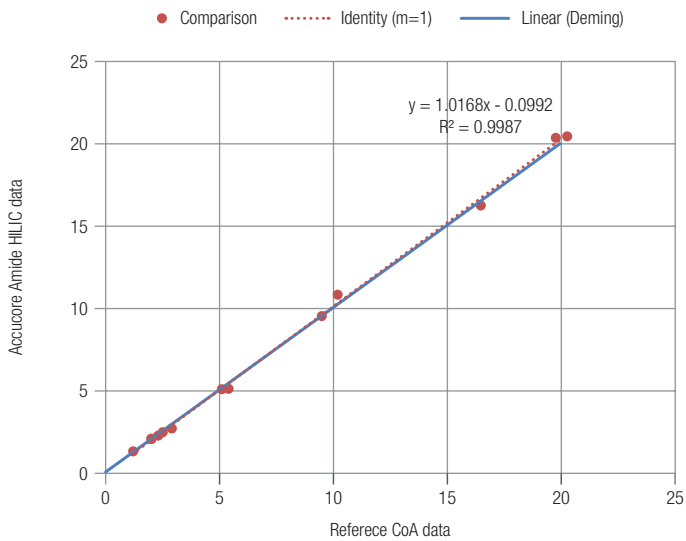
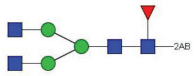
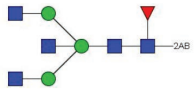
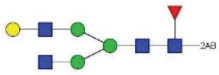
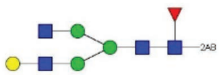
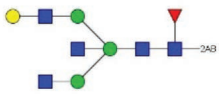
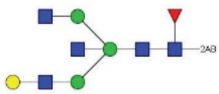
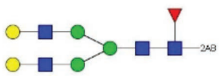
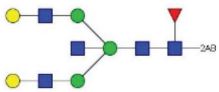
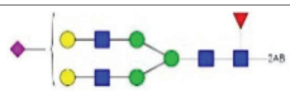
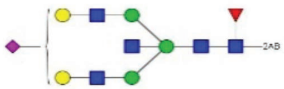
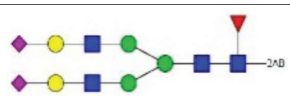
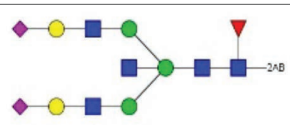


Figure 4. Correlation between manufacturer data and the test for the quantification of human IgG *N*-glycan. (The blue line indicates the theoretical identity of a perfect correlation; the red line is the plotted Deming regression with equation listed on the chart.)

Table 5. Accucore 150 Amide HILIC column (tested) vs. reference (manufacturer reported) in % relative peak area of 13 human IgG *N*-glycan

Peak ID	Full name	Short name	Structure	%relative area (reported)	%relative area (Tested)	%BIAS
1	F(6)A2	FA2		19.8	20.4	-3%
2	F(6)A2B	FA2B		5.4	5.08	6%
3	F(6)A2[6]G(4)1	FA2G1		20.3	20.4	-1%
4	F(6)A2[3]G(4)1	FA2G1		9.5	9.5	0%
5	F(6)A2[6]BG(4)1	FA2BG1		5.1	5.0	1%
	F(6)A2[3]BG(4)1	FA2BG1				
6	F(6)A2G(4)2	FA2G2		16.5	16.2	2%
7	F(6)A2BG(4)2	FA2BG2		2.9	2.7	9%
8	A2G(4)2S1	A2G2S1		2.5	2.6	3%
9	F(6)A2G(4)2S1	FA2G2S1		10.2	10.8	3%
10	F(6)A2BG(4)2S1	FA2BG2S1		2.3	2.2	4%
11	A2G(4)2S2	A2G2S2		1.2	1.3	-5%
12	F(6)A2G(4)2S2	FA2G2S2		2	2.0	-2%
13	F(6)A2BG(4)2S2	FA2BG2S2		2	2.0	0%

Conclusions

Glycan separation and characterization has remained as an important and challenging aspect of biotherapeutic characterization. The Accucore 150 Amide HILIC columns coupled to a Vanquish UHPLC system formed a robust platform and an excellent choice for glycan separations. Chromatograms showed great peak shape and resolution, at a moderate column backpressure. Additionally, the superb lot-to-lot column reproducibility and column lifetime demonstrate the consistency and robustness of the column. Finally, the accurate and precise quantification of *N*-glycan of human IgG illustrates the impressive correlation with a fully validated manufacturing quantification method, which provides a robust and confident reference for biotherapeutics QbD approach and quality control validation.

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Acknowledgement

We thank our Thermo Fisher Scientific colleagues Jim Thayer and Anahita Eckard for gererous assistance with glycan samples. We thank Jonathan Bones, Principal Investgator, NIBRT for comments that greatly improved the manuscript.

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